

Amendments to the Specification

On the first page of the specification, please replace paragraph 1 (at line 3) with the following replacement paragraph:

1. CROSS-REFERENCE TO RELATED CO-PENDING APPLICATIONS

This application claims benefit of priority under 35 U.S.C. § 119(e) to application no. 60/431,156, December 4, 2002 and application No. 60/525,284, entitled "MULTIPLEX AMPLIFICATION OF POLYNUCLEOTIDES," filed November 25, 2003 (~~attorney docket no. P-71902-1~~), the disclosure of which is incorporated herein by reference.

On page 4 of the specification, please replace paragraph 2 (at line 10) with the following replacement paragraph:

It has also been discovered that the presence of oligonucleotide probes in the multiplex amplification does not significantly interfere with the amplification reactions. Thus, the multiplex amplification can be effectively carried out in the presence of oligonucleotide probes, such as, for example, non-priming oligonucleotide probes designed for quantitative or real-time PCR analysis. Non-limiting examples of types of probes that can be present in the multiplex amplification include ~~TaqMan~~ TAQMAN® probes (see, e.g., U.S. Pat. No. 5,538,848), stem-loop or hairpin ~~Molecular Beacons~~ MOLECULAR BEACONS™ (see, e.g., U.S. Pat. Nos. 6,103,476 and 5,925,517 and Tyagi & Kramer, 1996, Nature Biotechnology 14:303-308), stemless or linear beacons (see, e.g., WO 99/21881), PNA Molecular Beacons (see, e.g., U.S. Pat. Nos. 6,355,421 and 6,593,091), linear PNA beacons (see, e.g., Kubista et al., 2001, SPIE 4264:53-58), non-FRET probes (see, e.g., U.S. Pat. No. 6,150,097), ~~Sunrise®/Amplifluor®~~ SUNRISE®/AMPLIFLUOR® probes (U.S. Pat. No. 6,548,250), stem-loop and duplex-~~Scorpion~~ SCORPION™ probes (Solinas et al., 2001, Nucleic Acids res. 29: E96 and U.S. Pat. No. 6,589,743), bulge loop probes (U.S. Pat. No. 6,590,091), pseudo knot probes (U.S. Pat. No. 6,548,250), cyclicons (U.S. Pat. No. 6,383,752), ~~MGB-Eclipse~~ MGB ECLIPSE™ probe (Epoch Biosciences), hairpin probes (U.S. Pat. No. 6,596,490), peptide nucleic acid (PNA) light-up probes, self-assembled nanoparticle probes, and ferrocene-modified probes.

On page 6 of the specification, please replace paragraph 1 (at line 8) with the following replacement paragraph:

The accumulation of single-plex amplification product can be monitored at the end of the reaction by conventional means, e.g., by chromatography, by eletrophoresis, by staining or by the use of a sequence specific hybridization probe (e.g., a fluorescently labeled probe). Alternatively, the accumulation of single-plex amplification product can be monitored as a function of time using well known methods, such as carrying out the single-plex amplification in the presence of one or more dyes or labels capable of producing a detectable signal upon binding double-stranded polynucleotide (e.g., SYBR® Green I or II, SYBR® Gold, ethidium bromide, or YO-PRO-1; Molecular Probes, Eugene, OR) or an oligonucleotide probe labeled with a suitable labeling system (e.g. a ~~TaqMan~~ TAQMAN® probe, or one of the various different types of exemplary probes described above).

On page 6 of the specification, please replace paragraph 2 (at line 19) with the following replacement paragraph:

The present invention also provides reagents and kits suitable for carrying out the multiplex amplifications and optional downstream analyses. In one embodiment, the kit includes a plurality of amplification primer sets suitable for carrying out a multiplex amplification packaged in a single container. The kit may optionally include one or more additional reagents for carrying out the amplification, such as a DNA polymerase enzyme, a reverse transcriptase enzyme and/or mixtures of nucleoside triphosphates ("dNTPs") suitable for extension of the primers via template-dependent DNA synthesis. The amount of optional polymerase included in the kit may be suitable for optimizing the efficiency of the multiplex amplification reaction. The various reagents may be packaged in combinations for maximal convenience, and may be modeled after the combinations of reagents available commercially for carrying out conventional PCR and/or RT-PCR amplification reactions (e.g., (2×) ~~TaqMan~~ TAQMAN® Universal PCR Master Mix and ~~TaqMan~~ TAQMAN® Gold RT-PCR Kit available from Applied Biosystems, an Applera Corporation business). The kit may further include reagents useful for carrying out downstream assays or analyses with the multiplex amplification product. For example, the kit may further include oligonucleotide probes useful for SNP detection or analysis, oligonucleotide microarrays, such as microarrays suitable for gene expression or SNP analyses, and/or "tailed" primers (see, e.g., Bengra et al., 2002, Clin.

Chem. 48:2131-2140; Myakishev et al., 2001, Genome Res. 11:163-169; and U.S. Pat. No. 6,395,486) for universal amplification, detection and/or purification. In one embodiment, the kit further includes reagents suitable for carrying out a plurality of single-plex quantitative or real-time amplification reactions. Such reagents typically include a set of quantitative or real-time amplification primers, an oligonucleotide probe labeled with a labeling system suitable for monitoring the quantitative real-time amplification reaction, a DNA polymerase at a concentration suitable for single-plex amplification and/or mixtures of dNTPs suitable for template-dependent DNA synthesis. The kit may include one or more of any of these additional reagents.

On page 8 of the specification, please replace paragraph 4 (at line 16) with the following replacement paragraph:

FIG. 4 provides a graph illustrating the observed amplification efficiency of an embodiment of a 95-plex amplification reaction of the invention carried out with a total of 100 ng cDNA (from a cDNA library) and 6 U/20 μ L ~~AmpliTaq-Gold~~ AMPLITAQ GOLD® for a total of 10 cycles (cycle time was approx. 1 min./cycle);

On page 13 of the specification, please replace paragraph 2 (at line 23) with the following replacement paragraph:

The number of sequences that may be amplified, or, stated another way, the number of amplicons that may be generated, by a multiplex amplification is dictated in large part by the number of different amplification primer pairs used during the multiplex amplification. According to certain embodiments of the invention, each amplification primer pair includes two amplification primers, one forward amplification primer and one reverse amplification primer, as is well-known in the art. The amplification primer pairs may be sequence-specific and may be designed to hybridize to sequences that flank a sequence of interest to be amplified. Thus, the actual nucleotide sequences of each primer pair may depend upon the sequence of interest to be amplified, and will be apparent to those of skill in the art. Methods for designing primer pairs suitable for amplifying specific sequences of interest via PCR or RT-PCR are well-known. See e.g., Eckert et al. (1991) PCR: A Practical Approach, McPherson, Quirke, and Taylor eds., IRL Press, Oxford, Vol. 1, pp. 225-244; TaqMan TAQMAN® Universal PCR Master Mix Protocol (available from Applied Biosystems, an

Applied Biosystems Corporation business, Cat. # 4304449 Rev. C); Rozen et al., 2000, *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Humana Press, Totowa, N.J., pp 365-386; <http://www.ucl.ac.uk/wibr/2/services/reldocs/taqmanpr.pdf>; <http://www.ukl.uni-freiburg.de/core-facility/taqman/taqindex.html>; <http://www.operon.com/oligos/toolkit.php>; http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; <http://www.ncbi.nlm.nih.gov/BLAST/>; and <http://www.biotech.uiuc.edu/primer.htm>, which provide examples demonstrating how particular primer pairs may be designed.

On page 17 of the specification, please replace paragraph 4 (at line 21) with the following replacement paragraph:

As will be described in more detail below, the product of a multiplex amplification can be used in a variety of different downstream assays and/or analyses. In a specific embodiment that will be discussed further, below, the product of a multiplex amplification reaction may be used in a plurality of subsequent, single-plex ("simplex"), quantitative or real-time PCR amplification reactions, such as for example, the quantitative or real-time amplifications routinely employed for gene expression analysis and which are commonly known in the art as 5'-exonuclease assays or ~~TaqMan~~ TAQMAN® assays (see, e.g., U.S. Pat. No. 5,691,146). When the product of the multiplex amplification reaction is to be used in this manner, the number of and/or sequences of the amplification primer pairs utilized in the multiplex amplification reaction can be correlated to correspond to the number of downstream single-plex, quantitative amplification reactions that may be performed. For example, if 96 downstream single-plex 5'-exonuclease amplification assays are desired, then the multiplex amplification can be carried out with a pool of 96 different sets of amplification primers or pairs. Correlating the number of amplification primer pairs with the number of subsequent single-plex quantitative amplification reactions is particularly convenient or advantageous in embodiments in which each subsequent single-plex quantitative amplification reaction will be carried out with a pair of amplification primers identical in sequence to one of the pairs used in the multiplex amplification reaction.

On page 18 of the specification, please replace paragraph 3 (at line 19) with the following replacement paragraph:

As discussed above, depending upon the nature of the sample polynucleotides to be amplified (e.g., RNA or DNA), a multiplex amplification reaction can be accomplished by polymerase chain reaction (PCR) or reverse-transcription PCR (RT-PCR). Thus, multiplex amplifications in which the target polynucleotide(s) is a DNA will typically include as essential components, in addition to the plurality of amplification primer pairs or sets discussed above, a mixture of 2'-deoxyribonucleoside triphosphates suitable for template-dependent DNA synthesis (e.g., primer extension) and a DNA polymerase. Multiplex amplifications in which the target polynucleotide(s) is a RNA will typically additionally include a reverse-transcriptase. With the exception of certain parameters described below, and the use of a plurality of amplification primer pairs instead of a single pair as described above, the multiplex amplification reactions may be carried out using reagents, reagent concentrations and reaction conditions conventionally employed in such conventional PCR and RT-PCR reactions. For example, except as noted herein, the various different primer concentrations, enzymes (e.g. DNA polymerases and reverse transcriptases), enzyme concentrations, dNTP mixtures (as well as their absolute and/or relative concentrations), total target polynucleotide concentrations, buffers, buffer concentrations, pH ranges, cycling times and cycling temperatures employed in conventional PCR and RT-PCR reactions may be used for the multiplex amplification reactions described herein. Guidance for selecting suitable reaction conditions may be found, for example, in U.S. Pat. Nos. 4,683,202; 4,683,195; 4,800,159; 4,965,188; 5,561,058; 5,618,703; 5,693,517; 5,876,978; 6,087,098; 6,436,677; and 6,485,917, and PCR Essential Data, J. W. Wiley & Sons, Ed. C. R. Newton, 1995, and PCR Protocols: A Guide to Methods and Applications. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990), all of which are incorporated herein by reference. A variety of tools for designing PCR and RT-PCR amplification primers, as well as myriad protocols, reaction conditions and techniques for carrying out various different types of PCR reactions, including conventional PCR reactions and RT-PCR reactions are also available online (see, e.g., http://www.protocol-online.org/prot/Molecular_Biology/PCR/index.html). All of these various tools and protocols can be used in connection with the multiplex amplification reactions described herein.

On page 19 of the specification, please replace paragraph 1 (at line 18) with the following replacement paragraph:

Like conventional PCR and RT-PCR amplification reactions, the multiplex amplification reactions may be carried out with a variety of different DNA polymerases (or mixture of DNA polymerases), but are preferably carried out in the presence of one or more thermostable polymerases. Suitable thermostable polymerases include, but are not limited to, Taq and Tth (commercially available from Applied Biosystems, an Applera Corporation business). Moreover, like conventional RT-PCR amplification reactions, multiplex RT-PCR amplification reactions may be carried out with a variety of different reverse transcriptases (or mixture of reverse transcriptases), although in some embodiments thermostable reverse-transcriptions are preferred. Suitable thermostable reverse transcriptases include, but are not limited to, reverse transcriptases such as AMV reverse transcriptase, MuLV, and Tth reverse transcriptase. Temperatures suitable for carrying out the various denaturation, annealing and primer extension reactions with the polymerases and reverse transcriptases are well-known in the art. Optional reagents commonly employed in conventional PCR and RT-PCR amplification reactions, such as reagents designed to enhance PCR, modify T_m , or reduce primer-dimer formation, may also be employed in the multiplex amplification reactions (see e.g., Pat. Nos. 6,410,231; 6,482,588; 6,485,903; and 6,485,944, all of which are incorporated herein by reference). In certain embodiments, the multiplex amplifications may be carried out with commercially-available amplification reagents, such as, for example, AmpliTaq AMPLITAQ® Gold PCR Master Mix, TaqMan TAQMAN® Universal Master Mix and TaqMan TAQMAN® Universal Master Mix No AmpErase AMPERASE® UNG, all of which are available commercially from Applied Biosystems, an Applera Corporation business.

On page 22 of the specification, please replace paragraph 2 (at line 16) with the following replacement paragraph:

Typically, conventional PCR and RT-PCR reactions are carried out with 0.05 U/ μ L DNA polymerase. For a 10 cycle 95-plex amplification carried out with 1 U/20 μ L AmpliTaq Gold AMPLITAQ GOLD® DNA polymerase (Applied Biosystems, an Applera Corporation business), it has been found that adding an additional 1-8 U/20 μ L increases the efficiency of the multiplex amplification. As will be described in more detail in the Examples section, significant increases in efficiency were observed with an additional 1-5 U/20 μ L AmpliTaq Gold AMPLITAQ GOLD® DNA polymerase. Increases were also observed with an additional 6-15 U/20 μ L, but they were less pronounced, potentially, due to the additional glycerol added to the reaction mixture as a result of spiking the reaction mixture with AmpliTaq Gold

AMPLITAQ GOLD® DNA polymerase stored in 50% glycerol. (see, e.g., FIG. 3). Similar increases in efficiency are also expected for other DNA polymerases, such as, for example, TaqI polymerase, Klenow fragment of DNA polymerase I, SEQUENASE 1.0 and SEQUENASE 2.0 (U.S. Biochemical), T5 DNA polymerase and Phi29 DNA polymerase. Thus, in one embodiment, a multiplex amplification is carried out in the presence of from about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 Units DNA polymerase per every 20 µL of reaction volume.

On page 29 of the specification, please replace paragraph 4 (at line 33) with the following replacement paragraph:

An embodiment of a matched or correlated multi-step assay, which can be created using the Assays-On-Demand ASSAYS-ON-DEMAND® service available from Applied Biosystems, available at the on-line website product's section, ~~(see e.g.,~~ http://www.appliedbiosystems.com/products/productdetail.cfm?prod_id=1101) is illustrated in ~~FIG. 2. Referring to FIG. 2, there, where~~ a plurality of 5'-exonuclease amplification primer/probe sets are selected by the user and pooled together to yield a plurality of amplification primer pairs or sets suitable for multiplex amplification (the pool also includes the plurality of 5'-exonuclease probes). A separate aliquot of each of the selected 5'-exonuclease amplification primer/probe sets are dispensed into individual reaction vessels, such as the wells of a multiwell plate, a single primer/probe set per vessel or well. In a first step, target polynucleotides from a sample of interest are multiplex amplified in the presence of the pooled amplification 5'-exonuclease primers/probes. The product of the multiplex amplification is then aliquoted into the wells of the multiwell plate, and single-plex 5'-exonuclease amplification assays are carried out using conventional methods. In one particularly convenient embodiment, the 5'-exonuclease primer/probe sets may be dispensed among the wells of a micro fluidic card that can be used directly on an instrument designed for quantitative or real-time amplification analysis, such as the AB Prism 7900 HT instrument available from Applied Biosystems (an Applied Biosystems Corporation business). An example of a suitable microcard is described in U.S. Pat. No. 6,126,899 and a commercial embodiment is the 7900HT Micro Fluidic card available from Applied Biosystems (an Applied Biosystems Corporation business).

On page 31 of the specification, please replace paragraph 3 (at line 21) with the following replacement paragraph:

In one specific embodiment, oligonucleotide probes present in a multiplex amplification are suitable for monitoring the amount of amplicon(s) produced as a function of time. Such oligonucleotide probes include, but are not limited to, the 5'-exonuclease assay (TaqMan TAQMAN®) probes described above (see also U.S. Pat. No. 5,538,848), various stem-loop molecular beacons (see, e.g., U.S. Pat. Nos. 6,103,476 and 5,925,517 and Tyagi & Kramer, 1996, Nature Biotechnology 14:303-308), stemless or linear beacons (see, e.g., WO 99/21881), PNA Molecular Beacons MOLECULAR BEACONS™ (see, e.g., U.S. Pat. Nos. 6,355,421 and 6,593,091), linear PNA beacons (see, e.g. Kubista et al., 2001, SPIE 4264:53-58), non-FRET probes (see, e.g., U.S. Pat. No. 6,150,097), Sunrise®/Amplifluor® SUNRISE®/AMPLIFLUOR® probes (U.S. Pat. No. 6,548,250), stem-loop and duplex Scorpion SCORPION™ probes (Solinas et al., 2001, Nucleic Acids res. 29: E96 and U.S. Pat. No. 6,589,743), bulge loop probes (U.S. Pat. No. 6,590,091), pseudo knot probes (U.S. Pat. No. 6,548,250), cyclicons (U.S. Pat. No. 6,383,752), MGB-Eclipse MGB ECLIPSE™ probe (Epoch Biosciences), hairpin probes (U.S. Pat. No. 6,596,490), peptide nucleic acid (PNA) light-up probes, self-assembled nanoparticle probes, and ferrocene-modified probes described, for example, in U.S. Pat. No. 6,485,901; Mhlanga et al., 2001, Methods 25:463-471; Whitcombe et al., 1999, Nat. Biotechnol. 17:804-807; Isacsson et al., 2000, Mol. Cell. Probes. 14:321-328; Svanvik et al., 2000, Anal Biochem. 281:26-35; Wolffs et al., 2001, Biotechniques 766:769-771; Tsourkas et al., 2002, Nucleic Acids Res. 30:4208-4215; Riccelli et al., 2002, Nucleic Acids Res. 30:4088-4093; Zhang et al., 2002, Shanghai. 34:329-332; Maxwell et al., 2002, J. Am. Chem. Soc. 124:9606-9612; Broude et al., 2002, Trends Biotechnol. 20:249-56; Huang et al., 2002, Chem. Res. Toxicol. 15:118-126; and Yu et al., 2001, J. Am. Chem. Soc. 123:11155-11161, all of which are incorporated herein by reference.

On page 33 of the specification, please replace paragraph 2 (at line 12) with the following replacement paragraph:

In carrying out a multiplex amplification in the presence of such a dye molecule, or in the presence of suitable oligonucleotide probes, Applicants have discovered a general method for characterizing pooled sets of primers. In the method, the amplification is monitored in real time, and a cycle threshold value ("Ct^{pool}") obtained. This is an additive

signal produced by the summation of all of the amplicons. In the embodiment in which oligonucleotide probes are used for the real time multiplex amplification, a separate probe is present for each target sequence being amplified, and all of the probes use the same signaling system. The method is especially useful in providing a rapid and convenient test of pooled reagents that may be provided in ready-made, pre-optimized, kits. Such a pool of reagents can be prepared by mixing commercially available primer sets such as the ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND® Gene Expression products or the primer sets available in the QuantiTect Gene Expression Assays (Qiagen), as further described herein.

On page 35 of the specification, please replace paragraph 3 (at line 27) with the following replacement paragraph:

Also provided herein are reagents and kits suitable for carrying out the multiplex amplification and various two-step reactions and/or assays described herein. Such reagents and kits may be modeled after reagents and kits suitable for carrying out conventional PCR and RT-PCR amplification reactions, with the exception that instead of a single set of amplification primers, the reagents and/or kits include a plurality of amplification primers packaged in a single container, wherein the single container may additionally contain one or more oligonucleotide probes, as described herein. Examples of specific reagents include, but are not limited, to the reagents present in ~~Assays-by-Design~~ ASSAYS-BY-DESIGN™, ~~Pre-Developed Assay Reagents (PDAR)~~ for gene expression, ~~PDAR~~ for allelic discrimination and ~~Assays-On-Demand~~ ASSAYS-ON-DEMAND®, which are commercially available at Applied Biosystems (an Applied Biosystems Corporation business). The kits may optionally include reagents packaged for downstream or subsequent analysis of the multiplex amplification product. In one embodiment, the kit includes a container comprising a plurality of amplification primer pairs or sets, each of which is suitable for amplifying a different sequence of interest, and a plurality of reaction vessels, each of which includes a single set of amplification primers suitable for amplifying a sequence of interest. The primers included in the individual reaction vessels can, independently of one another, be the same or different as a set of primers comprising the plurality of multiplex amplification primers. In a specific embodiment, both the container and plurality of reaction vessels further include 5'-exonuclease probes such that the kit is suitable for carrying out the multistep assay illustrated in FIG. 2. In one embodiment, the plurality of reaction vessels is a multiwell plate.

On page 36 of the specification, please replace paragraph 2 (at line 22) with the following replacement paragraph:

To determine the optimal amount of DNA polymerase for performing multiplex amplifications, 95-plex amplifications were carried out as a function of DNA polymerase concentration. The amplification primer mix for the 95-plex amplification was prepared by pooling 10 μ L from each of 95 different randomly selected 20 \times Assays-on-Demand ASSAYS-ON-DEMAND[™] Gene Expression Products (Applied Biosystems, an Applied Biosystems Corporation business, Catalog Nos. Hs0170531_m1; Hs00176369_m1; Hs00176332_m1; Hs00170586_m1; Hs00173565_m1; Hs00176247_m1; Hs00170192_m1; Hs00177127_m1; Hs00176908_m1; Hs00170380_m1; Hs00173925_m1; Hs00170681_m1; Hs00176394_m1; Hs00170633_m1; Hs00173872_m1; Hs00174690_m1; Hs00170288_m1; Hs00173798_m1; Hs00170423_m1; Hs00174927_m1; Hs00174805_m1; Hs00175976_m1; Hs00176222_m1; Hs00173678_m1; Hs00170261_m1; Hs00173592_m1; Hs00174781_m1; Hs00177401_m1; Hs00173854_m1; Hs00173936_m1; Hs00170248_m1; Hs00173564_m1; Hs00174717_m1; Hs00170407_m1; Hs00174575_m1; Hs00174796_m1; Hs00176315_m1; Hs00170969_m1; Hs00153126_m1; Hs00174765_m1; Hs00153510_m1; Hs00173606_m1; Hs00176075_m1; Hs00170236_m1; Hs00170712_m1; Hs00176239_m1; Hs00176121_m1; Hs00171022_m1; Hs00170174_m1; Hs00173506_m1; Hs00174910_m1; Hs00170210_m1; Hs00174789_m1; Hs00174774_m1; Hs00173773_m1; Hs00174937_m1; Hs00173681_m1; Hs00170903_m1; Hs00176268_m1; Hs00176148_m1; Hs00176865_m1; Hs00174599_m1; Hs00170308_m1; Hs00170823_m1; Hs00176077_m1; Hs00173899_m1; Hs00174860_m1; Hs00173717_m1; Hs00175940_m1; Hs00170684_m1; Hs00173526_m1; Hs00170299_m1; Hs00170991_m1; Hs00176385_m1; Hs00175935_m1; Hs00170403_m1; Hs00173855_m1; Hs00170899_m1; Hs00176202_m1; Hs00170349_m1; Hs00177051_m1; Hs00170472_m1; Hs00173634_m1; Hs00175948_m1; Hs00177552_m1; Hs00175997_m1; Hs00174752_m1; Hs00174674_m1; Hs00176505_m1; Hs00176209_m1; Hs00175999_m1; Hs00176998_m1; Hs00176747_m1; Hs00170433_m1; and Hs00174604_m1. Each 20 \times Assays-on-Demand ASSAYS-ON-DEMAND[™] Gene Expression Product contained two unlabeled amplification primers (18 μ M each primer) and one FAM-labeled TaqMan TAQMAN[®] MGB probe (5 μ M). 95-Plex amplifications were carried out with this amplification primer mix using DNA polymerase concentrations ranging from 1 Unit per 20 μ L reaction volume (1 U/20 μ L) to 17 U/20 μ L. For the 95-plex amplification carried out with 1 U/20 μ L DNA polymerase, 5 μ L pooled primer mix, 10 μ L 2 \times TaqMan TAQMAN[®] Universal PCR Master Mix ("2 \times Master Mix"; Applied

Biosystems, an Applied Biosystems Corporation business, Cat. #4304437) and 5 µL template cDNA (from a cDNA library; 100 ng total cDNA) were added to a reaction tube. 2× Master Mix comprises ~~AmpliTaq-Gold~~ AMPLITAQ GOLD® DNA polymerase (0.1 U/µL), AmpErase[®] UNG, dNTPs with dUTP, a passive reference and optimized buffer components. 95-Plex amplifications carried out at higher DNA polymerase concentrations were prepared by spiking the reaction with the appropriate amount of ~~AmpliTaq-Gold~~ AMPLITAQ GOLD® (5 U/µl; Applied Biosystems Catalog No. N808024). All 95-plex reactions were initially heated (10 min at 95° C.) followed by a total of 10 cycles (15 sec melt at 95° C.; 1 min anneal/extend at 60° C.) on an ~~ABI-Prism~~ ABI PRISM® 7700 instrument (Applied Biosystems, an Applied Biosystems Corporation business).

On page 38 of the specification, please replace paragraph 1 (at line 3) with the following replacement paragraph:

The product of each 95-plex amplification was diluted to 200 µl with water (10-fold) and divided for 95 individual single-plex real-time amplification reactions. Each single-plex amplification used as primers/probes one of the 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND™ Gene Expression Products described above, with a different set of primers per reaction. The following volumes of reagents were used for the single-plex real-time amplifications: 2 µL diluted 95-plex amplification product, 1 µL 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND™ Gene Expression Product, 10 µL 2× Master Mix and water to yield a 20 µL reaction volume. All single-plex amplifications were carried out for a total of 40 cycles (using the same cycling conditions as described above) on an ~~ABI-Prism~~ ABI PRISM® 7700 or 7900 instrument (Applied Biosystems, an Applied Biosystems Corporation business). The accumulation of amplicon was monitored in real time. These amplifications are the “assay amplifications.”

On page 39 of the specification, please replace paragraph 3 (at line 27) with the following replacement paragraph:

Another significant advantage of multiplex amplifications is the ability to carry out the reaction in the presence of oligonucleotide probes without significant interference during either the multiplex amplification or downstream amplifications carried out on the multiplex amplification product. This former advantage is apparent from Example 2, supra. In Example

2, efficient amplification was achieved in the multiplex amplification step, which by virtue of utilizing ~~Assays-On-Demand~~ ASSAYS-ON-DEMAND[™] reagents to create the multiplex primer pool, included ~~TaqMan~~ TAQMAN[®] MGB oligonucleotide probes in the reaction.

On page 41 of the specification, please replace paragraph 1 (at line 2) with the following replacement paragraph:

To demonstrate that multiplex amplifications can be carried out at very high levels of complexity, 186-plex, 369-plex, 738-plex and 1013-plex amplifications were carried out in four individual multiplex amplification reactions. The amplification primer mix for each of the amplifications was prepared by pooling equal volumes of 186, 369, 738 or 1013 different randomly selected 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND[™] Gene Expression Products into four separate microcentrifuge tubes, respectively. For each of the four tubes, the pooled solution was dried using a ~~SpeedVae~~ SPEEDVAC[®] concentrator (Thermo Savant, Holbrook, N.Y.). The residue was re-suspended in deionized water such that the multiplexed amplification primers were at a 4×stock concentration (180 nM each primer) relative to the 1×working amplification primer concentration of 45nM. For the 1013-plex pooled mixture, the combined primers were present in the re-suspension at a concentration of 45.6 μM, and the FAM-labeled ~~TaqMan~~ TAQMAN[®] MGB probes were present at 10.1 μM. For the 186-plex amplification, 92 primer sets from each of two plates (designated IAP and IAO) were pooled along with equal volumes of primer sets for two reference genes, glyceraldehyde phosphate dehydrogenase (GAPDH) and cyclophilin. In setting up the experiment described in this Example, for convenience in liquid transfers, each of the above randomly selected 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND[™] Gene Expression Products was distributed into a series of 96-well plates (designated alphabetically plates IAA through IAO). Each 20× ~~Assays-on-Demand~~ Gene Expression Product contained two unlabeled amplification primers (18 μM each primer) and one FAM-labeled ~~TaqMan~~ TAQMAN[®] MGB probe (5 μM).

On page 41 of the specification, please replace paragraph 2 (at line 23) with the following replacement paragraph:

Each of the amplifications (from the 186-, 369-, 738- or 1013-plex pooled primer mixtures) were carried out in a final volume of 50 μL, with the constituents being 12.5 μL of 4×pooled and re-suspended primer mix, 25 μL 2× ~~TaqMan~~ TAQMAN[®] Universal PCR Master

Mix ("2× Master Mix"; Cat. #4324016 containing no UNG enzyme), 10 µL template cDNA (from a cDNA library; 25 ng total cDNA) and 2.5 µL ~~AmpliTaq Gold~~ AMPLITAQ GOLD® DNA polymerase (5U/µL). The 2× Master Mix included ~~AmpliTaq Gold~~ AMPLITAQ GOLD® DNA polymerase (0.1 U/µL), dNTPs, a passive reference and optimized buffer components. Each of the four reactions were carried out for a total of 10 cycles (15 sec. melt at 95° C.; 4 min. anneal/extend at 60° C.) on an ~~ABI-Prism~~ ABI PRISM® 7700 instrument.

On page 41 of the specification, please replace paragraph 3 (at line 32) with the following replacement paragraph:

The product of each amplification was diluted with water and aliquoted for single-plex analysis. In the case of the 186- and 369-plex reactions, the product was diluted 1:5 prior to setting up the single-plex assays. For the 738- and 1013-plex amplifications, the product was diluted 1:10 prior to setting up the single-plex assays. Each of these single-plex amplifications used as primers/probes one of the 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND™ Gene Expression Products used in the multiplex amplification described above, and were distributed into a series of 96-well plates for liquid transfer convenience (designated alphabetically plates IAA through IAO). A different set of primer/probes was used in each single-plex reaction. The following volumes of reagents were used for the single-plex ("assay") amplifications: 2.5 µL diluted 186-, 369-, 738- or 1013-plex amplification product, 0.5 µL 20× ~~Assays-on-Demand~~ ASSAYS-ON-DEMAND™ Gene Expression Product, 5 µL 2× Master Mix and water to yield a 10 µL reaction volume. All assay amplifications were carried out for a total of 40 cycles (15 sec. melt at 95° C.; 1 min. anneal/extend at 60° C.) on an ~~ABI Prism~~ ABI PRISM® 7900 instrument. The accumulation of amplicon was monitored in real time.

On page 43 of the specification, please replace paragraph 2 (at line 11) with the following replacement paragraph:

A first 186-plex amplification (UNG(-)) was carried out as described in Example 5, but using ~~TaqMan~~ TAQMAN® Universal Master Mix, No AmpErase AMPERASE® UNG (Cat. #4324018), instead of Universal Master Mix (Cat. #4304437). The multiplex amplification was extended for 14 cycles, instead of 10 cycles, as in Example 5. The samples were chilled on ice after the amplification, and then subjected to single-plex PCR as described in Example 5.